

SUPPLEMENTARY FILE

S2.1a Thermogravimetric analysis (TG)

Thermogravimetric analysis using Perkin Elmer TG/DTA analyzer (STA 6000, Massachusetts, U.S.A) operating in the range of 40°C to 740°C with at temperature rise of 10.00°C/min and flow of nitrogen gas is 100ml/min.

S2.1b Stability studies of silver nanoparticles (AgNPs)

To investigate the physical and chemical stability of nanoparticle suspension, samples were stored at $(30 \pm 2^{\circ} \text{C}$ and $65 \pm 5 \text{ RH}$) and $(5 \pm 3^{\circ} \text{C})$ in accordance to the *ICH guidelines [Q1A(R2)]* for long term storage of general and drug substances intended for storage in a refrigerator respectively. Absorbance max, size and zeta potential of the prepared nanoparticles were determined as the parameters for defining stability.

S2.2 Synthesis, optimization and characterization of copper-curcumin (Cu-Cur) and copper-curcumin- β -Cyclodextrin (Cu-Cur)CD inclusion complex

25ml methanolic solution of cupric acetate (0.220 g, 4 mmol) was added into the 13.5 ml of methanolic solution of curcumin (0.185 g, 2mmol). Dark reddish brown precipitates were produced immediately. The mixture was refluxed for 2 hr under a nitrogen atmosphere. The solid product was then filtered, washed with cold methanol and water to remove the residual reactants, and then the product was dried in vacuum overnight. The compound is characterized by UV absorption spectroscopy (UV-Visible spectrophotometer, Shimadzu, Japan), IR (FTIR Spectrophotometer, Nicolet-380, Thermo, USA), $^1\text{H-NMR}$ (Bruker Avance II 400 NMR) and DSC (Mettler Toledo DSC 822e).

Inclusion complex was prepared by solvent evaporation encapsulation method; with slight modifications as reported in the previous research.(Yallapu et al., 2010) (β -CD) 100 mg was dissolved in 20mL deionized water in a 50 mL beaker containing a magnetic bar. (Cu-Cur) complex in different concentrations, (10-50)% dissolved in 1 ml tetrahydrofuran (THF) was added to (β -CD) solution under stirring at 600-800 rpm. Stirring was done for 12hr in dark with a perforated aluminum foil covering for acetone evaporation. Highly water soluble (Cu-Cur)CD was separated from the supernatant after centrifugation at 1500 rpm and then recovered by lyophilizer (Alpha 1-2

LD plus, Martin Christ, Germany). Maximum (Cu-Cur) loading inside (β -CD) cavity was optimized by assessing the per mg (Cu-Cur) loading using DMSO based extraction method. Optimized inclusion complex was characterized by IR, $^1\text{H-NMR}$, morphology (Scanning electron microscope, JSM- 840 SEM, Jeol, Japan) and DSC.

Results

S3.1a Thermogravimetric analysis (TG)

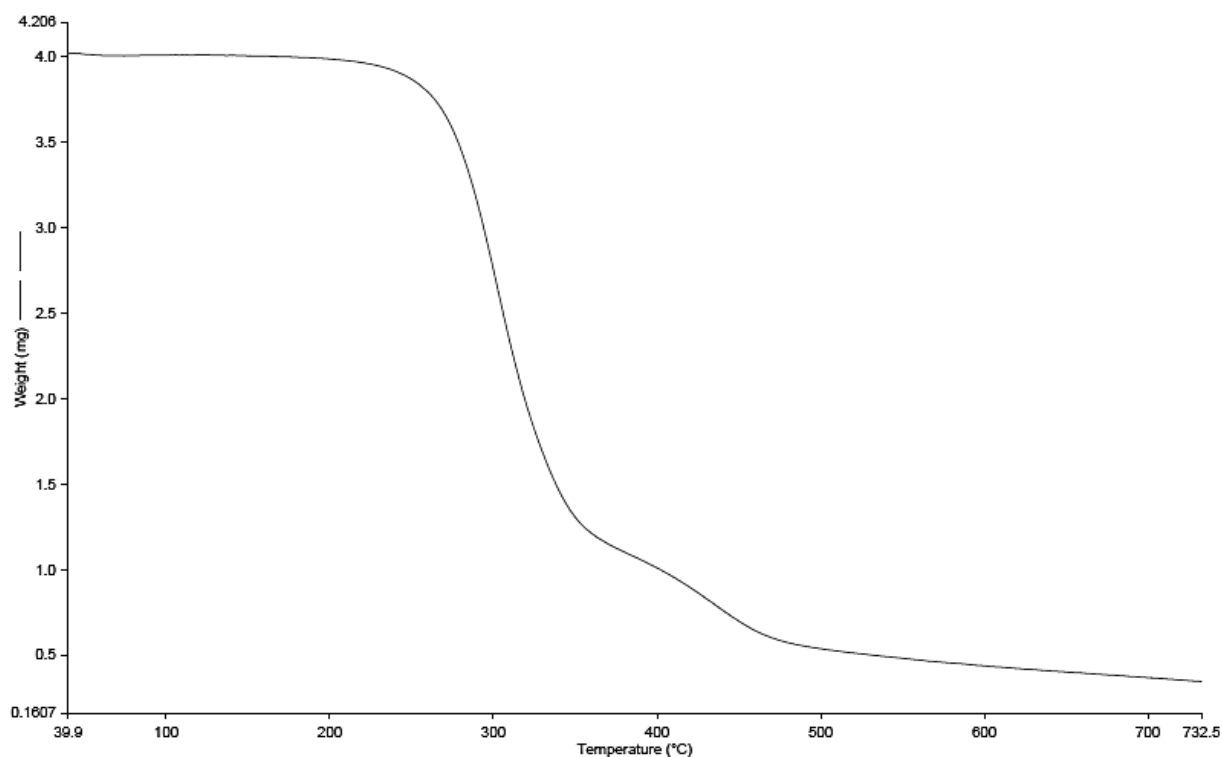


Figure-1s 4 TG themogram of in-situ stabilized silver nanoparticles.

S2.2 Synthesis, optimization and characterization of copper-curcumin (Cu-Cur) and copper-curcumin- β -Cyclodextrin (Cu-Cur)CD inclusion complex

Reddish brown colored (Cu-Cur) complex was obtained and this synthesized complex was characterized for various parameters. UV spectral study in DMSO revealed a single peak for Cur at 432 nm and two peaks for Cu-Cur complex at 433 and 456 nm. IR (KBr) cm^{-1} : spectral data of

curcumin and (Cu-Cur) reveals the formation of new bands at 535cm^{-1} and 477cm^{-1} in (Cu-Cur) spectra indicating the interaction between Copper(II) and oxygen (O) atom of curcumin.(Kolev et al., 2005) $^1\text{H-NMR}$ (DMSO_d): spectral data of curcumin and(Cu-Cur)revealed the absence of characteristic peaks of curcumin (6 – 8 ppm) in the spectra of the metal complex, indicating the chelation with the metallic ion. DSC thermogram showed a sharp peak for the melting of (Cu-Cur) around 197.3°C , which indicates the formation of crystalline metal complex.

(Cu-Cur)CD inclusion was prepared according to the procedure mentioned and further it was optimized on the basis of loading of (Cu-Cur) inside the β -CD hydrophobic cavity. (Cu-Cur) loading was estimated for each batch of inclusion complex. Table-I explains the preparation and optimization of inclusion complex. Study revealed that lyophilized batch (MC-40) and MC-50 have shown comparatively higher (Cu-Cur) loading (around $24\mu\text{g}/\text{mg}$ of complex) and % yield (88-90mg). Rationally, formulation (MC-40) was considered as optimum for further characterization and other studies.

(Cu-Cur)CD complex (MC-40) appears as light greenish yellow colored fluffy powder. This fluffy appearance points toward its amorphous nature. Aqueous saturation solubility of the (MC-40) at ambient conditions was found to be around $38.5 \pm 1.4 \text{ mg}/\text{ml}$. UV spectra in deionized water showed an absorption maxima at 265nm .

IR (KBr) cm^{-1} spectra revealed the major existence of (β -CD) specific peaks with a slight shift to higher/lower wave numbers while only few characteristic peaks of (Cu-Cur) were observed. $^1\text{H-NMR}$ spectra again displayed the same dominance as (MC-40) showed all the peaks relevant to (β -CD). But inclusion of (Cu-Cur) results in the shifting of proton signals to high field region.

DSC thermograms are indicative of complexation of (Cu-Cur) within cyclodextrin cavity. DSC thermograms of (MC-40) complexes showed a substantial reduction in the intensity of the peak, broadening of peak and a shift to lower temperature (136 to 143°C) in comparison to sharp melting point of pure (Cu-Cur). Marked reduction in intensity accompanied by broadening and shift to a lower temperature of the endotherm indicates the substantial inclusion of (Cu-Cur) in the (β -CD) cavities. Scanning electron microscopy clearly justifies the above observation of having a highly amorphous nature of (MC-40).

S2.7.1 In-vitro challenge study for the pharmaceutical parameters of NHM gel

A three-factor, three-level Box-Behnken statistical experimental design requires 17 experiments. The independent variables and the responses for all 17 experimental runs are given in Table-1(sup)

Table-1s Analysis of Response 1, 2 and 3 (i.e viscosity at 37°C), pH, mucoadhesion and spreadibility.

	Factor 1	Factor 2	Factor 3	Response 1	Response 5	Response 6	Response 7
Run	A:Gel g	B:VSF ul	C:SS ml	viscosity-37	pH	spreadibility	mucoadhesion
1	2	5500	6	0.689	6.72	17	21
2	3	3000	2	0.332	5.4	34	42
3	3	5500	4	0.674	5.96	26	35
4	3	3000	6	0.67	5.8	22	32
5	3	500	4	0.372	5.29	37	46
6	1	3000	2	0.209	5.88	8	17
7	2	3000	4	0.358	5.66	19	28
8	2	3000	4	0.358	5.66	21	29
9	1	5500	4	0.346	6.8	7	16
10	2	3000	4	0.358	5.66	15	24
11	2	500	6	0.61	7.07	18	29
12	2	500	2	0.343	6.4	24	33
13	2	3000	4	0.358	5.66	18	26
14	2	5500	2	1.319	5.29	17	28
15	2	3000	4	0.358	5.66	14	23
16	1	500	4	0.275	6.89	11	17
17	1	3000	6	0.602	6.5	8	13

- **Analysis of viscosity**

This analysis is analysis is better explained by contour plots and response surface analysis as mentioned in figure- 4.24.1, 4.24.2.

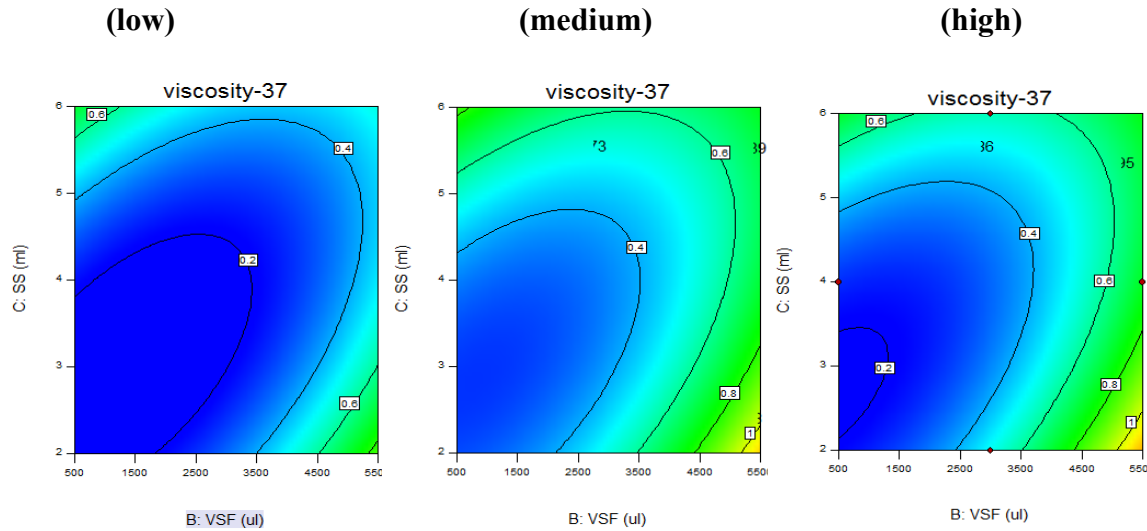


Figure-2as Contour plots showing the interaction effect. (Blue region is low viscosity (<0.5 region))

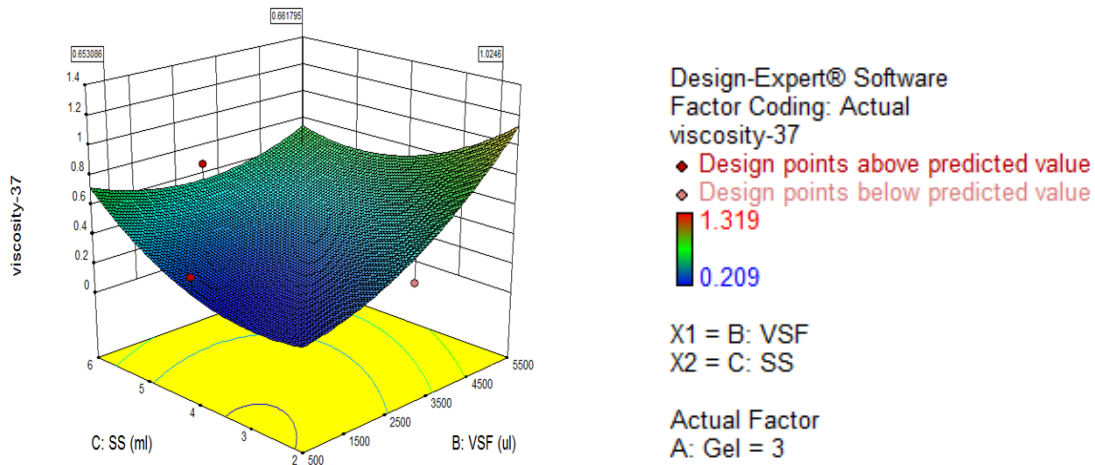


Figure-2bsResponse surface plot showing effect of VSF (B) and SS (C) interaction with high level of gel (A) on response (Viscosity- 37°C)

- Analysis of pH

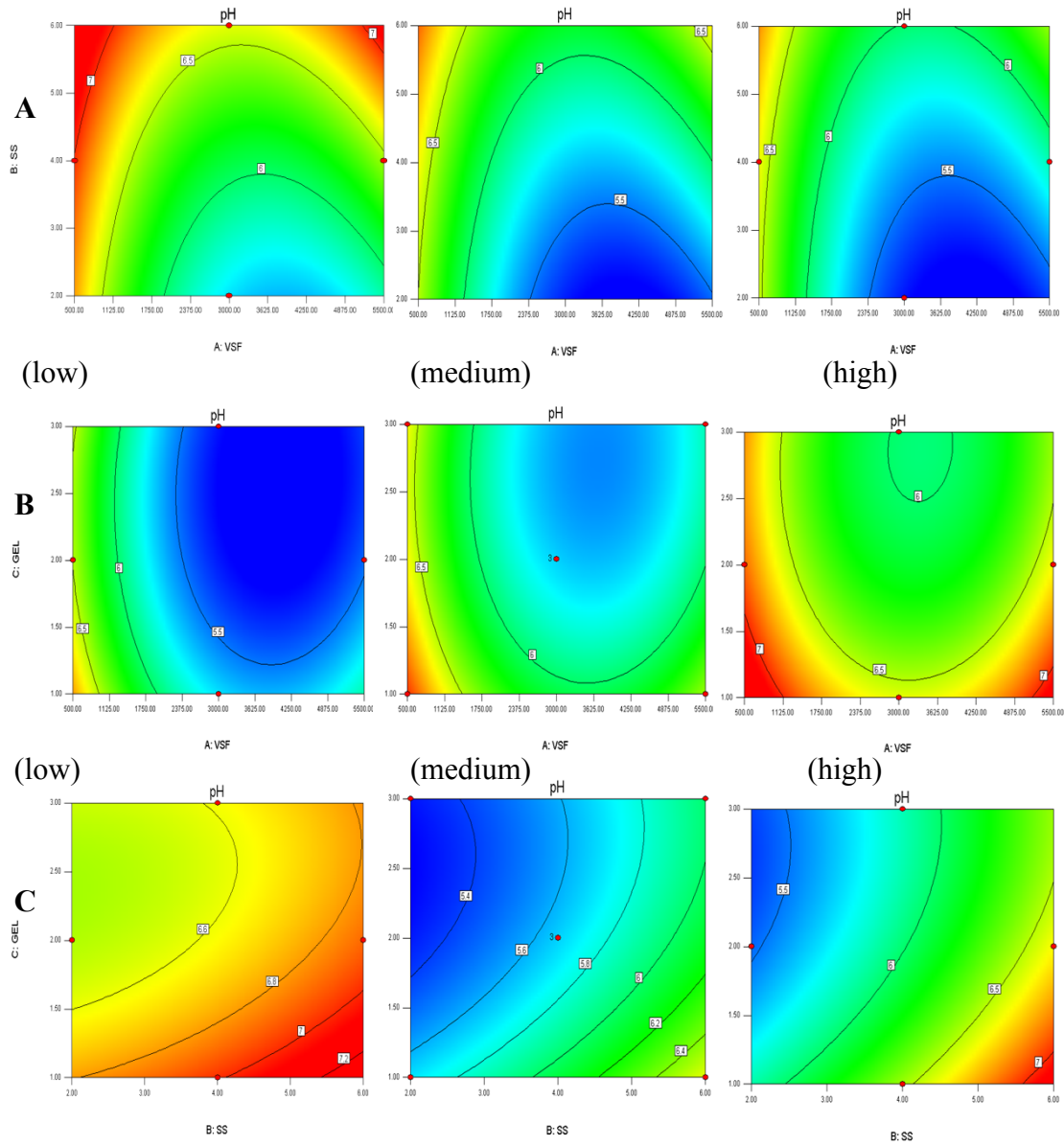


Figure-3s Contour plots showing the interaction effect. (Blue region is low pH (<math><5.8</math> region))

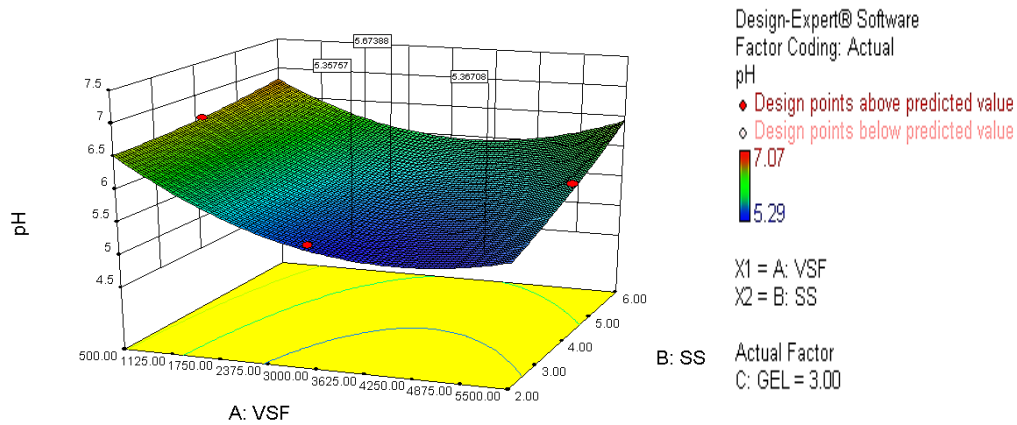


Fig-4s Response surface plot showing effect of VSF (A) and SS (B) interaction with high level of gel (C) on response (pH).

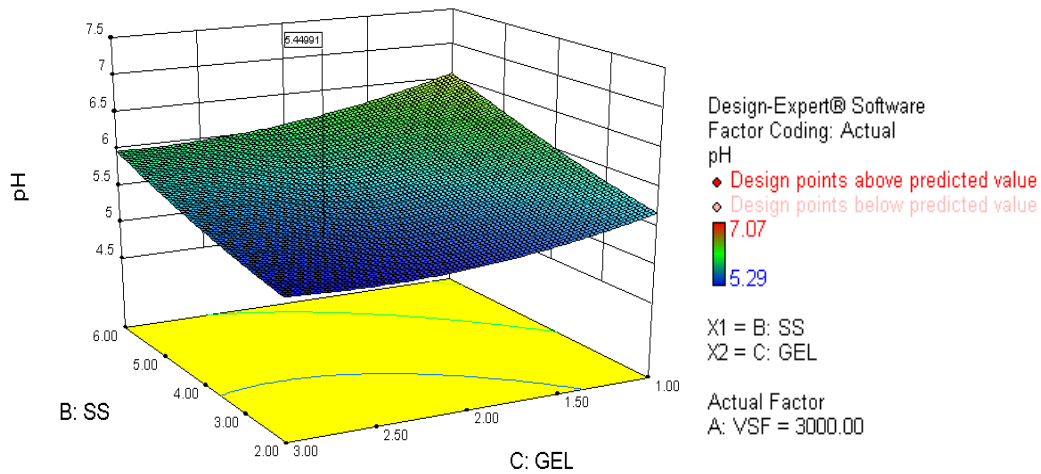


Fig-5s Response surface plot showing effect of Gel (C) and SS (B) interaction with medium level of VSF (C) on response (pH).

- **Analysis of spreadability**

Spreadability characteristic of a microbicide gel is very important from the fact that complete covering of the vaginal mucosa is required.

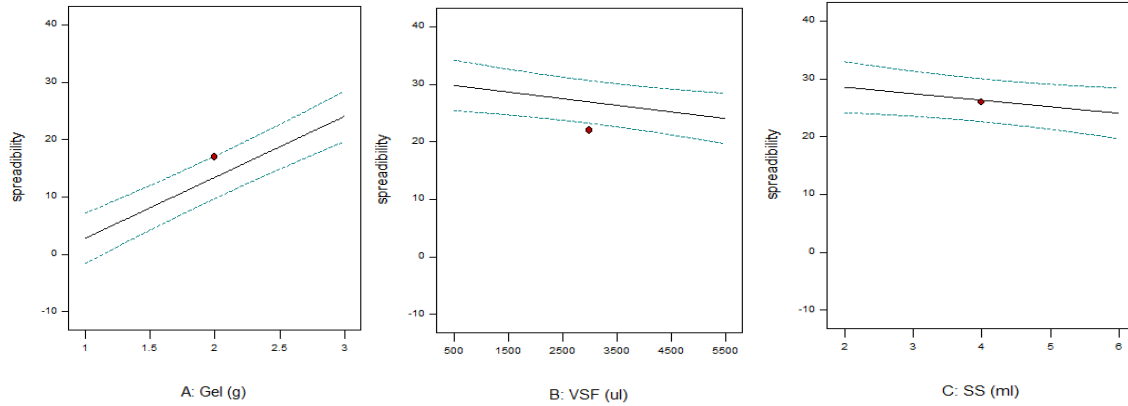


Figure-6s Contour plots showing the interaction effect (for spreadability)

- **Analysis of mucoadhesion:**

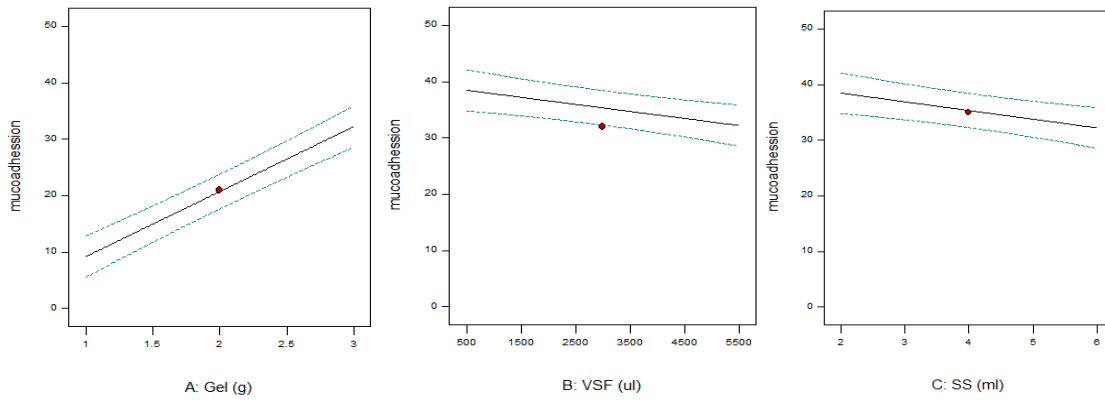


Figure-7s Contour plots showing the interaction effect (for mucoadhesion)

2.6 Anti-Candida Activity

Minimum inhibitory concentration (MIC) was assessed for *C. albicans* and *C. tropicalis* using serial dilution assay using 96-well flat-bottomed microtiter plate.

- Strains and preparation of inocula

C. albicans (Microbial Type Culture Collection (MTCC-183) and *C. tropicalis* (Microbial Type Culture Collection (MTCC-184) strains were used in this study. The strains were inoculated onto sabouraud dextrose agar plates and incubated at 35 °C for 24 h. They were then subcultured on the same medium for a further 24 h at 35 °C. The fungal inocula were prepared by diluting the overnight culture with 0.9% NaCl to $1-5 \times 10^6$ CFU/mL. The suspensions were then further diluted in RPMI-1640 (with L-glutamine, without sodium bicarbonate) supplemented with 0.165M morpholinepropanesulfonic acid (MOPS) to a final count ranging 0.5×10^3 CFU/mL.

- Minimum inhibitory concentration

The antifungal effectiveness was determined against the final cell concentration of 10^6 CFU/mL for both *C. albicans* and *C. tropicalis* cells. Fungal inocula (100 μ L) were added to each well of a sterile, 96-well flat-bottomed microtiter plate. Arithmetic concentrations (in 100 μ L volume) of AgNPs (2-200) μ g/ml and (Cu-Cur)CD (2-20)mg were added methodically in the wells. Each concentration was tested in triplicate for each fungal strain. 300 μ L of deionized sterile water were added on the exterior perimeter wells of the microplates to minimize the evaporation of the culture medium of the test wells during the incubation. For each strain two wells containing fungal suspension with no drug (growth control) and 2 wells containing only media (background control) were included in that plate. ODs were measured for 24 h at 35°C using a microplate reader (Bio-Rad) at 405nm. Turbidimetric growth curves were obtained depending on the changes in the OD of fungal growth for each drug concentration and the drug-free growth control. MICs value for AgNPs and (Cu-Cur)CD using this assay (the growth percentage for each drug concentration) was calculated with the below equation. OD measurement were reported only after the subtraction of background ODs (ODs of fungal cells free wells).

$$\text{Growth \%} = [(\text{OD}_{405} \text{ of wells containing the test} / \text{OD}_{405} \text{ of the drug free well}) \times 100]$$